Assessing a Training Screen Employing Image-based Cellular Assays –
Antagonist GPCR assays in live-cells with Cypher5E™

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Introduction
The implementation of image-based cellular assays in high-throughput screening presents a unique set of challenges, from assay development and optimization to through evaluation of screen quality. We have performed a case study based on antagonist format screens of commercial training libraries using a live-cell Cypher5E™ GPCR internalization assay.

Cypher5E™ is a red-excited pH sensitive fluorescent dye that has been designed to be minimally fluorescent at basic pH and maximally fluorescent at an acidic pH. It is therefore ideal for monitoring the translocation of cell surface receptors into the endosomal pathway. The assay system is based on the incubation of a Cypher5E-conjugated anti-VEG-FG antibody with a clonal stable HEKA 293 cell line expressing N-terminally epitope tagged (2-Adrenergic) receptor. In response to agonist stimulation, a fluorescent signal is observed when the Cypher5E-β-adrenergically conjugate is internalised into the endoctic endosomal vesicles. Dose-response and screening work is performed using 4 replicate control plates that have been designed to be minimally fluorescent at basic pH conditions.

Screening at the optimal concentration gave the following results (responses were normalized to the controls on each plate where agonist control = 100%):

1) Image Analysis
By using the image analysis capabilities of the algorithms developed for the IN Cell Analyzer instruments (specifically the GRN1 algorithm) and adjusting secondary analysis filtering one can re-analyse the images to identify and characterise other modes of possible interaction that may lead to false-positives eg cytotoxic effects. Images that may indicate toxic effects are shown.

2) Z'AMP Measurement
To confirm specificity we ran the 61 hits in the assay with the replacement of the Cypher antibody addition step with media alone. After the agonist incubation step we a) decanted and added 10μl 1% lysis buffer or b) added 20μl 10% lysis buffer (duplicate plates were set up). The supernatants (10μl and 4μl respectively) were then transferred to a 384-well plate and assayed for cAMP using a radiometric cAMP assay utilizing LEADseeker PS beads. The assay was measured on the LEADseeker Multimodality imaging system.

The results of the Z'AMP measurements are shown:  

All 8 hits from the 100nM Cypher5E screen were confirmed by Z'AMP-50pM assay. These 8 antagonists were all known 2-Adrenergic receptor antagonists. Of the remaining 7 confirmed hits some were adrenergic receptor ligands but not of the subclass; others were dopamine or 5HT ligands which may act as an additional structurally related compound.

Conclusions
• Live cell GPCR antagonist screening can be accomplished using Cypher5E™ and the IN Cell Analyzer 1000 system.
• Optimizing the library concentration can improve screen quality.
• Z' AMP is useful in assessing assay quality on each plate.
• Performing the screen at 100nM offers an option of providing very specific hits in this screen.
• Performing a screen at 10μM gave more hits, but the ‘hits’ were less specific for the B2AR.
• Image acquisition and analysis can potentially automate false hit identification.
• Higher content cellular assays present challenges but also opportunities for screening with regard to optimizing the library concentration to be more selective.